Increased Urinary Phosphate Excretion Induced by Fibroblast Growth Factor 23 in Zinc-deficient Rats

Toru Mochizuki1, Takashi Miyazaki1, Tsuneo Takenaka3 and Hiromichi Suzuki1, 2

1 Community Health Science Center Saitama Medical University*, 2 Department of Nephrology, Faculty of Medicine Saitama Medical University*, 3 Sanno Hospital, Clinical Research Center of International University of Health and Welfare**

Summary

Introduction: Zinc (Zn) is an essential trace element in cellular metabolism and skeletal mineralization. It is well known that 90% of Zn in the entire body is found in muscle and bone. Fibroblast growth factor 23 (FGF23) is a key regulator of Pi homeostasis and contributes to several hypophosphatemic disorders through decreased expression of the Na-Pi cotransporter type IIa (Na/Pi IIa) and Na/Pi IIC on the renal cortical brush border membrane. Whether FGF23 regulates Pi metabolism in Zn-deficient (ZnD) rats is unclear. Therefore, we measured the serum levels of FGF23 and 1,25-dihydroxy vitamin D3 (1,25-VD3) in ZnD rats, as well as the mRNA levels of bone FGF23, renal Klotho, Na/Pi IIa and Na/Pi IIC. Methods: The bone mineral density (BMD) of the femur and urinary Pi excretion estimated using a dual X-ray absorption method. Control groups consisted of rats on a paired calorie Zn-sufficient diet (P), and rats that were allowed to feed freely (F). Results: Compared with the P group, the serum levels of 1,25-VD3 in the ZnD group were significantly reduced. Bone FGF23 mRNA expression, serum levels of FGF23, and urinary Pi excretion were significantly increased compared with the P group. The expression of Na/Pi IIC was significantly decreased compared with the F groups. Expression of Klotho mRNA was inhibited by Zn deficiency. Conclusion: These results suggest that Zn deficiency may inhibit 1,25-VD3 synthase, resulting in increased serum FGF23. Moreover, increased FGF23 may reduce the reabsorption of Pi, resulting in a decrease in BMD.

Keywords: Fibroblast growth factor 23, Urinary phosphate excretion, Bone mineral density, Sodium/phosphate co-transporters, Zinc-deficient rat.

Abbreviations: Zn, zinc; Pi, phosphate; Ca, calcium; Cre, creatinine; 1,25-VD3, 1,25-(OH)2-vitamin D3; ZnD, zinc-deficiency; BMD, bone mineral density; Na/Pi IIa, sodium/phosphate cotransporter type IIa; PTH, parathyroid hormone; FGF 23, fibroblast growth factor 23; FEP, fractional excretion of phosphate.

Introduction

Zinc (Zn) is an essential trace element that is an important nutrient cofactor of numerous enzymes1, transcription factors5, and an intracellular signaling mediator6, 7. Physiological Zn is a cofactor of more than 300 enzymes, and more than 2,000 Zn-dependent proteins involved in stabilizing protein structures. Zn deficiency leads to anorexia, loss of appetite, failure of smell and taste, human growth retardation, and alopecia and osteoporosis in animals8, 9. It is important to realize that Zn deficiency is a major worldwide health problem with more than 30% of the global population at risk, predominantly in underdeveloped countries5, 9, 10.

Of the Zn in the whole body, 90% is contained in muscle and bone11. Bone is composed of inorganic mineral hydroxyapatite, which includes calcium (Ca), phosphate (Pi) and Zn. Metabolism of Ca and Pi in bone is strictly regulated by 1,25-(OH)2-vitamin D3 (1,25-VD3) and parathyroid hormone (PTH). Increased PTH secreted from the parathyroid gland leads to an elevated serum Ca level, reduced serum Pi level, but increased urinary excretion of Pi, indicating that the balance of Ca and Pi represents an inverse relationship12, 13.

Shimada et al.14 cloned cDNA from a hemangiopericytoma that caused hypophosphatemic osteomalasia and

* Address: 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan
** Address: 8-10-16 Akasaka, Minato-ku, Tokyo, 107-0052 Japan

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found clones identical to fibroblast growth factor 23 (FGF23), which has been identified by positional cloning as a gene responsible for autosomal dominant hypophosphatemic rickets. They reported that FGF23 is secreted from osteocytes and osteoblasts. CHO-FGF23 tumors implanted into nude mice induced severe hypophosphatemia, growth retardation, rickets in the growth plates, deformities of the skeleton and reduced mineralization of bone. These bone disorders are related to Pi over-secretion from the kidney, suggesting an induced reduction of bone mineral density (BMD). Renal Pi excretion and reabsorption is mediated primarily by the sodium/Pi cotransporter type IIa (Na/Pi IIa) and Na/Pi IIc. FGF23 protein binds to the FGF receptor, bound by the transmembrane protein Klotho as a coreceptor. Klotho was discovered by Kuro-o et al. They indicated that the Klotho-knockout mouse induces aging. Another report indicated that PTH increases Pi excretion through FGF23 and Klotho. Therefore, the regulation of Pi in Zn-deficient rat may be affected by FGF23 production.

In this study, the effect of Zn deficiency on FGF23, PTH, 1,25-VD₃, mRNA Klotho expression, Pi metabolism through Na/Pi IIa and Na/Pi IIc, and BMD were evaluated.

Materials and Methods

Chemicals

All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Sigma-Aldrich (St Louis, MO, USA).

Animals, and urine, serum and tissue sample preparations

All animal experiments were approved by the Animal Research Committee of Saitama Medical University (Approval number: 25M072). Pathogen-free, male, Sprague-Dawley rats (mean ± SD, 106 ± 0.8 g, n = 30) were purchased from Tokyo Laboratory Animal Science Co. (Tokyo, Japan). The rats were acclimatized in individual cages in a temperature-controlled room (22-24°C) with a 12-hour (h)/12-h light/dark cycle for 1 week. The rats were then divided into three groups and fed either a Zn-deficient diet (ZnD group, n = 10), a Zn-sufficient diet (5 mg/100 g Zn) that was calorie paired with the ZnD group (Pf group, n = 10), or ad libitum (F group, n = 10) for 4 weeks, as reported previously. The amounts of Zn in the diet of Pf rats were determined the day before from the ZnD-fed-rat level. After 4 weeks, the sera, kidneys and femurs were obtained, and the samples were frozen in liquid nitrogen for subsequent real-time PCR analysis of FGF23, Klotho and Na/Pi II transporters. For evaluation of urinary excretion of bone minerals, the BMD level of the femur was also determined. The obtained sera were used for determinations of Zn, FGF23, 1,25-VD₃, PTH, Ca, Pi and creatinine (Cre).

Collection of urine samples and determinations of urinary Ca, Pi and Cre

To determine urinary secretion levels of Ca, Pi and Cre, urine samples were obtained using a KN-646 metabolic cage (Natsume Seisakusyo, Tokyo, Japan) over a course of 15 h (6 PM to 9 AM). The urine samples were stored at −80°C until analyzed. The urinary concentrations of Ca, Pi and Cre were measured using a commercial kit (Ca for Calcium C-test, Pi for Phosphor C-test and Cre for Creatinine test; Wako Pure Chemical Inc., Osaka, Japan). The urinary levels of Pi and Ca were expressed as µg excreted Pi or Ca per 15 h per 100-g body weight.

Real-time PCR analysis

Real-time polymerase chain reaction (PCR) was performed using a Bio-Rad iQcycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). For real-time PCR analysis of FGF23, Klotho, Na/Pi-IIa and Na/Pi-IIc, total RNA was obtained from the kidney and femur. Bone marrow was washed from the femur with saline, and the remaining thin bone was used for total RNA extraction. Total RNAs were extracted using ISOGEN (Nippon Gene, Tokyo, Japan), and cDNA was synthesized from the extracted total RNA using an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories), following the manufacturer’s protocol. The FGF23 primers (accession number; AB078777): Forward (F): 5’-ttggatcgtatcacttcagc-3’, Reverse (R): 5’-tgcttcggtgacaggtag-3’, the Klotho primers (AB017820), F: 5’-ggccgcaccttcagggattac-3’, R: 5’-atcgggcagcagggatgaga3’, Na/PiIIa primers (NM_013030); F: 5’-agcacctcgacatccatcat-3’, R: 5’-cggagctcacctccaaca-3’, Na/Pi IIc primers (NM_139338); F: 5’-agctgaagaatactgaccaactca-3’, R: 5’-gaccacctggtgcagctt-3’, and GAPDH (AF_106860); F: 5’-aaacccatcaccatcttcca3’, R: 5’-gtggttcacacccatcacaa-3’ were used for PCR. The synthesized cDNA, the specific primers and iQ™SYBR™ Green Supermix (Bio-Rad Laboratories) were used for PCR, using the manufacturer’s recommended conditions (94°C for 30 s, 60°C for 30 s, and 72°C for 60 s; 40 cycles). The results of the PCR amplification were compared with those of the GAPDH, and data were presented by 2⁻ΔΔCT values as relative fold-expression levels.
Determination of serum FGF23, PTH, and 1,25-VD₃

Serum levels of FGF23, PTH and 1,25-VD₃ were determined for each group at 4 weeks after implementation of the diet. The detection limits of the assay were 1 pg/ml for PTH (electrochemiluminescence immunoassay, ECLIA), 5 pg/ml for 1,25-VD₃ (radioimmunoassay with two antibodies), and 3 pg/ml for FGF23 (ELISA), as reported previously.

Determinations of serum Pi, Ca, and Cre

To determine the serum levels of Ca, Pi and Cre, sera were obtained at 4 weeks after implementation of the diets. Blood samples were obtained from the abdominal aorta under deep ether anesthesia. The samples were then centrifuged at 3,000 rpm for 10 min, and the separated sera were used for determination of serum Ca, Pi and Cre. The concentrations of Pi, Ca and Cre in serum samples were determined with commercial kits (Wako Pure Chemical) using an Ultramark Micro Imaging System (Bio-Rad) microplate reader. The serum levels of Ca and Pi were expressed as mg/dl.

The fractional excretion of Pi (FEP) was calculated as:

\[
FEP = \frac{\text{urinary [Pi]} \times \text{serum [Cre]}}{\text{urinary [Cre]} \times \text{serum [Pi]}}
\]

Determination of BMD in the femur

For determination of BMD, the femur was obtained under deep ether anesthesia. The soft connective tissues and muscle were removed, and then the BMD of the cleaned bone was measured by DXA using a Lunar PIXI-mus densitometer (GE Healthcare Co., CT, USA). TriPLICATE measurements were made from each of six independent samples from each group.

Statistical analysis

Statistical analyses were performed using the Kaleida Graph 4.0 software (Hulinks Inc., Tokyo Japan). All data shown represent means ± standard error (SE) or standard deviation (SD). The statistical significance of differences among groups was calculated by one-way ANOVA followed by Tukey’s test as a post hoc test. For all analyses, differences were considered to be significant at p-values < 0.05.

Results

Table 1 presents the serum Zn concentrations and body sizes of the rats prior to and at 4 weeks after implementation of the special diets. The serum level of Zn in the ZnD group was significantly diminished at 4 weeks, compared with the Pf and F groups as shown in Table 1. Characteristic alopecia of Zn deficiency was observed in 50% of the rats in the ZnD group (data not shown). There was no apparent diarrhea. From the rats’ movements, the ZnD group also appeared to be depressed. These characteristic symptoms of Zn deficiency, as reported previously, indicate that this rat model resulted in moderate Zn deficiency.

The relative fold levels (mean ± SE as 2^ΔΔCT values) of bone FGF23 mRNA were 1.51 ± 0.17 in the F group, 1.24 ± 0.18 in the Pf group and 2.85 ± 0.37 in the ZnD group compared to GAPDH, as shown in Fig. 1A. The FGF23 level in the ZnD group increased significantly compared to the Pf group (p = 0.0001), and ZnD group vs. F group (p = 0.001).

The serum levels of FGF23 in the ZnD group increased significantly, compared with the Pf group. The serum levels of PTH in the ZnD group were increased tendency (p = 0.0467 from the F group, p = 0.093 from Pf group). The serum level of 1,25-VD₃ in the ZnD group was decreased compared the Pf group (p = 0.046) as shown in Table 2.

There was no significant difference in the levels of serum Ca and Pi among the ZnD, Pf and F groups. In serum, the Ca concentration was 9.3 ± 0.8 mg/dl in the F group, 9.1 ± 0.7 mg/dl in the Pf group and 9.0 ± 0.8 mg/dl in the ZnD group, which were not significantly different. The serum levels of Pi were 7.2 ± 0.51 mg/dl in the F group, 9.1 ± 0.7 mg/dl in the Pf group and 9.0 ± 0.8 mg/dl in the ZnD group, which were not significantly different.

Table 1 Rat body weight and serum zinc level

<table>
<thead>
<tr>
<th></th>
<th>Pre-experiment (n = 10, g)</th>
<th>After 4 weeks (n = 10, g)</th>
<th>Serum zinc (n = 10, µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free group</td>
<td>176.7 ± 1.3</td>
<td>221.8 ± 1.6</td>
<td>118.5 ± 2.4</td>
</tr>
<tr>
<td>Pf group</td>
<td>175.4 ± 2.3</td>
<td>230.4 ± 12.8</td>
<td>107.3 ± 2.6</td>
</tr>
<tr>
<td>ZnD group</td>
<td>182.0 ± 1.6</td>
<td>180.8 ± 20.0</td>
<td>298 ± 40.0</td>
</tr>
</tbody>
</table>

Changes in rat body weight and serum Zn concentration. The groups had either free access to food (F), a paired-calorie Zn-sufficient diet (Pf), or a Zn-deficient diet (ZnD). The data are shown as means ± SE. The symbols (a) and (b) represent a significant difference (p < 0.01) between the ZnD group and the F and Pf groups, respectively.

Table 2 Serum FGF-23, PTH, and 1,25-Vitamin D₃ levels

<table>
<thead>
<tr>
<th></th>
<th>FGF-23 (n = 8, pg/ml)</th>
<th>PTH (n = 8, pg/ml)</th>
<th>1,25-VD₃ (n = 8, µg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free group</td>
<td>240.0 ± 9.8</td>
<td>6.05 ± 0.39</td>
<td>1773 ± 27.5</td>
</tr>
<tr>
<td>Pf group</td>
<td>230.0 ± 12.8</td>
<td>6.23 ± 0.29</td>
<td>230.0 ± 11.5</td>
</tr>
<tr>
<td>ZnD group</td>
<td>311.9 ± 201.1 a,b</td>
<td>7.35 ± 0.38 a,b</td>
<td>1822 ± 81.8</td>
</tr>
</tbody>
</table>

Serum levels of fibroblast growth factor 23 (FGF23), parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D₃ (1,25-VD₃). The groups had either free access to food (F), a paired-calorie Zn-sufficient diet (Pf), or a Zn-deficient diet (ZnD). The data are shown as means ± SE. The symbols (a) and (b) represent a significant difference (p < 0.05) between the ZnD group and the F and Pf groups, respectively.
mg/dl in the ZnD group, which were not significantly different.

The urine volume (ml/15 h/100 g body weight) was 1.44 ± 0.20 in the F group, 1.01 ± 0.24 in the Pf group and 1.67 ± 0.41 in the ZnD group in Fig. 2A. The urine volume level in the ZnD group decreased significantly compared with the Pf group (p = 0.0085). The level of excreted urinary Ca (µg/15 h/100 g body weight) was 61.6 ± 36.4 in the F group, 73.8 ± 85.4 in the Pf group and 25.2 ± 18.7 in the ZnD group. The level of excreted urinary Ca in the ZnD group was not significantly different compared with the Pf group.

The level of excreted urinary Pi (µg/15 h/100 g body weight) was 231.3 ± 71.9 in the F group, 3.3 ± 1.2 in the Pf group and 48.7 ± 11.6 in the ZnD group in Fig. 2B. The level of excreted urinary Pi in the ZnD group and Pf group was reduced significantly relative to the F group (p = 0.0063 and p = 0.0014, respectively), but the excreted Pi in ZnD group increased from the Pf group, which was not significantly different. The increased Pi reabsorption in Pf group compared with F group is physiological response, because the daily fed diet level of Pf group as same level of ZnD group is reduced (about 50-60% of F group) from F group fed level.

The FEP level in Pf group was significantly reduced from the F group (p = 0.0021) and the FEP level in ZnD group was increased significantly (p = 0.0093) from the Pf group (Fig. 4A).

Although there was no significant difference among the ZnD, Pf, and F groups, the relative fold increases in renal Na/Pi IIa mRNA expression were 1.32 ± 0.04 in the F group, 1.52 ± 0.19 in the Pf group and 1.80 ± 0.11 in the ZnD group, which were also not significantly different (Fig. 3A).

The relative fold increase in renal Na/Pi IIc mRNA expression was 2.17 ± 0.17 in the F group, 1.63 ± 0.09 in the Pf group and 1.41 ± 0.13 in the ZnD group; the Na/Pi IIc level in the ZnD group was significantly different from that in the F group (0.0066), but not the Pf group (Fig. 3B).

The BMD level of femurs in the ZnD group was significantly lower than in the Pf (p = 0.0032) and in the F (p = 0.0014) groups.
Osteocytes secrete sclerosin, and the FGF23 mRNA concentration in femurs increased significantly as a result of Zn deficiency in rats (Table 2). The relative fold increase in renal Klotho mRNA level was 12.0 ± 0.5 in the F group, 15.8 ± 2.2 in the Pf group, and 2.5 ± 0.4 in the ZnD group in Fig. 1B. The renal Klotho mRNA level was significantly lower in the ZnD group than in the F (p = 0.0040) and Pf (p < 0.0001) groups.

**Discussion**

The serum level of the bone-derived hormone FGF23 and the FGF23 mRNA concentration in femurs increased significantly as a result of Zn deficiency in rats (Table 2). These increased FGF23 levels apparently inhibited the renal reabsorption of Pi, leading to increased urinary excreted Pi and reduced BMD of the femur in the Zn-deficient rats. These results suggest that Zn deficiency induces FGF23 mRNA expression, which would elevate the serum level of FGF23, resulting in a decreased BMD in the femur.

Serum levels of FGF23 are mostly derived from osteocytes and osteoblasts in bone, suggesting that the relationship between these cells and Zn ions may be important. Osteocytes secrete sclerosin, an inhibitor of bone formation, the phosphaturic factor FGF23, as well as other gene products, such as phosphate-regulating endopeptidase on the X-chromosome (Phex) that regulate bone mineralization and FGF23 expression. The loss of Phex, an endopeptidase, by the Phex gene knockout model resulted in elevated circulating levels of FGF23 and inhibited renal Pi reabsorption in mice. Phex is a type I cell surface Zn metalloprotease, suggesting that Zn deficiency may reduce Phex activity. We speculate that the reduction of Phex inactivation by Zn deficiency may be related with increased serum FGF23 levels. Moreover, a Zn transporter, solute-carrier-39 (SLC39), belongs to a family whose members regulate Zn movement into the cytosol. Fukada et al. indicated that SLC39 knockout mice induce defects in the maturation of osteoblasts, indicating that the depletion of intracellular Zn concentration in osteoblasts/osteocytes may involve FGF mRNA expression. It is likely that Zn deficiency also induces the depletion of systemic Zn levels in the serum and cytosol, resulting in an increase in serum FGF23. Hie et al. reported that Zn deficiency in the rat reduced osteoblast differentiation and numbers through reductions in osteoblast specific transcription factor, rent-related transcriptional factor, Wnt signaling, and glycogen synthase kinase 3β. The depletion of these transcription factors by Zn deficiency may stimulate FGF23 transcription and elevate serum FGF levels.

Although the serum level of Pi was identical in both the ZnD and Pf groups, the level of excreted urinary Pi was greater in the ZnD group than in the Pf group. The Pi intake level from the diet and serum Pi level were identical in both the ZnD and Pf groups. Elevated FGF23 can stimulate urinary excretion of Pi through Na/Pi IIa and Na/Pi IIC. In our study, the renal mRNA level of Na/Pi IIC in Zn-deficient rats was significantly different from those of the F group rat, which is consistent with the report by Hu et al.

FGF23 is an important regulator of Pi homeostasis and vitamin D3 metabolism. An increased circulating level of FGF23 results in hypophosphatemia because of renal Pi wasting and a low serum level of 1,25-VD3. In this study, the serum level of 1,25-VD3 showed a tendency to decrease in Zn-deficient rats compared with the Pf group (Table 2). Furthermore, the activity of renal 1 α -hydroxylase is reduced by Zn deficiency, which may result in reduced synthesis of 1,25-VD3.

The serum Ca and Pi levels in ZnD rats were identical to those of Pf rats; nevertheless, the urinary excretion level of Ca in ZnD rats was lower than that in Pf rats (data not shown). Given that the balance of Pi and Ca is strictly controlled, increased renal excretion of Pi may induce
Therefore, Zn deficiency may inhibit 1,25-VD$_3$ synthase, resulting in elevated serum PTH, bone FGF23 mRNA, and serum FGF 23 levels. Furthermore, the increased serum FGF 23 level may reduce the reabsorption of Pi through Na/Pi IIa and Na/Pi IIc in the kidney, resulting in reduced femur BMD.

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